REMARKS

Claims 1-3, 5-13, 24-34 and 65-86 are pending in the application. All claims stand rejected.

Claims 28, 32, 69, 73, 76, 81 and 84 have presently been cancelled without prejudice or disclaimer. Applicants reserve the right to file one or more continuation, divisional and/or continuation-in-part applications directed to the cancelled subject matter and/or any other subject matter disclosed in the instant specification.

Claims 1, 6-13, 24, 27, 29, 31, 33, 34, 65, 68, 70, 72, 74, 75, 80, 83, 85 and 86 have been amended to more clearly describe and distinctly claim the subject matter the Applicants consider their invention. Specifically, claims 1, 6-13 and 65 have been amended to specify that R^1 and R^2 along with the adjacent atoms to which they are attached form an _optionally substituted_ phenyl group. Support for the amendments to claims 1, 6-13 and 65 can be found throughout the specification and claims as originally filed, _e.g._, page 8, lines 21-24, page 11, lines 23-29, Examples 51 and 52, and original claim 1. Claim 24 has been amended to delete the duplicated compound "(±)-Ethyl 2-ethoxy-3-[4-[[3-phenyl-4-oxo-3,4-dihydro-2-quinazolinyl]methoxy]phenyl]propanoate." Claims 29, 31, 33, 34, 70, 72, 74, 75, 83, 85 and 86 have been amended to correct minor typographical errors and to maintain proper dependency where necessary. Claims 27, 31, 68, 72, 80 and 83 have been amended to limit the method to treating or preventing diabetes or impaired glucose tolerance. No new matter has been introduced by the amendments to claims 1, 6-13, 24, 27, 29, 31, 33, 34, 65, 68, 70, 72, 74, 75, 80, 83, 85 and 86.

Claims 87-111 have been newly added. Claim 87 is directed to a method for the treatment or prophylaxis of disorders related to Syndrome X and corresponds to claims 29 and 70. Further support for claim 87 can be found throughout the specification as originally filed, _e.g._, page 45, line 11 to page 46, line 6. Claims 88-93 are directed to a method for the treatment or prevention of conditions associated with high blood glucose, high triglycerides and/or high total cholesterol. Support for claims 88-93 can be found throughout the specification as originally filed, _e.g._, page 90, line 24 to page 96, line 9. Claims 94-99 are directed to a method for the treatment or prevention of hyperlipidemia,

hypercholesterolemia, hyperglycemia, insulin resistance, obesity, leptin resistance and/or type II diabetes and correspond to diseases and conditions originally recited in amended claims 27, 31, 68, 72, 80 and 83 and cancelled claims 28, 32, 69, 73, 81 and 84. Further support for claims 94-99 can be found throughout the specification as originally filed, *e.g.*, page 45, line 11 to page 46, line 6 and page 90, line 24 to page 96, line 9. Claims 100-108 are directed to a method of activating PPARα and/or PPARγ in a cell. Support for claims 100-108 can be found throughout the specification as originally filed, *e.g.*, page 7, lines 11-17 and page 90, line 24 to page 96, line 9. Claims 109-111 are directed to a method for the treatment and/or prevention of a condition mediated by PPARα and/or PPARγ. Support for claims 109-111 can be found throughout the specification as originally filed, *e.g.*, page 4, line 11 to page 5, line 3, page 7, lines 11-17 and page 90, line 24 to page 96, line 9. No new matter has been introduced by new claims 87-111.

Claims 1-3, 5-13, 24-27, 29-31, 33, 34 and 65-68, 70-72, 74, 75, 77-80, 82, 83 and 85-111 are now in the case. Reconsideration and allowance of the claims in view of the amendments above and the remarks below are respectfully requested.

Claim Rejections – 35 U.S.C. § 112

Claims 1-3, 5, 24-34 and 66-76 are rejected under 35 U.S.C. § 112, second paragraph as allegedly indefinite because, according to the Examiner, the recitation of "its derivatives, its analogs, its tautomeric forms, its polymorphs, its pharmaceutically acceptable salts and its pharmaceutically acceptable solvates" is in incorrect Markush form. With respect to claims 28, 32, 69, 73 and 76, the claims have presently been cancelled. With respect to claims 1-3, 5, 24-27, 29-31, 33, 34 and 66-68, 70-72, 74 and 75, claim 1 has been amended to recite the remaining choices in alternate, singular form as per the Examiner's suggestion. Accordingly, reconsideration of this basis for rejection is respectfully requested.

Claims 1-3, 5, 24-34 and 66-76 are rejected under 35 U.S.C. § 112, second paragraph as allegedly indefinite because, according to the Examiner, the terms "derivatives" and "analogs" have unclear metes and bounds. With respect to claims 28, 32, 69, 73 and 76, the claims have presently been cancelled. With respect to claims 1-3, 5, 24-27, 29-31, 33, 34 and 66-68, 70-72, 74 and 75, claim 1 has been amended to delete

the terms "derivatives" and "analogs." Accordingly, reconsideration of this basis for rejection is respectfully requested.

Claims 1-13, 24-34 and 65-86 are rejected under 35 U.S.C. § 112, first paragraph as allegedly nonenabled because, according to the Examiner, the specification, while enabling for making pharmaceutically acceptable salts, does not reasonably provide enablement for making solvates and polymorphs. With respect to claim 4, the claim was previously cancelled by Amendment dated November 3, 2004. With respect to claims 28, 32, 69, 73, 76, 81 and 84, the claims have presently been cancelled. With respect to claims 8-13, 65, 77-80, 82, 83, 85 and 86, these claims do not recite the term "polymorphs" or "pharmaceutically acceptable solvates," either explicitly or through dependency. With respect to claims 1-3, 5-7, 24-27, 29-31, 33, 34, 66-68, 70-72, 74 and 75, claim 1 has been amended to delete the terms "polymorphs" and/or "pharmaceutically acceptable solvates." Accordingly, reconsideration of this basis for rejection is respectfully requested.

Claims 27-34 and 68-86 are rejected under 35 U.S.C. § 112, first paragraph as allegedly nonenabled because, according to the Examiner, the specification, while enabling for treating diabetes, does not reasonably provide enablement for treating or preventing any or all diseases or any or all cancers generically embraced in these claims. With respect to claims 28, 32, 69, 73, 76, 81 and 84, the claims have presently been cancelled. With respect to claims 27, 29-31, 33, 34, 68, 70-72, 74, 75, 77-80, 82, 83, 85 and 86, Applicants respectfully traverse this basis for rejection.

With respect to claims 77-79, these claims are directed to the compound (±)-Sodium 2-ethoxy-3-[4-[[3-methyl-4-oxo-3,4-dihydro-2-quinazolinyl] methoxy]phenyl] propanoate and a pharmaceutical composition comprising the same. None of these claims is directed to a method of treatment of any disease. As with claims 1, 25 and 26, and 24, 66 and 67 (which the Examiner did not reject under 35 U.S.C. § 112, first paragraph), Applicants submit that the specification enables these compound and compositions claims, and reconsideration of this basis for rejection is respectfully requested.

42

With respect to claims 30, 34, 71, 75, 82 and 86, these claims are limited to reducing plasma glucose, triglycerides, total cholesterol, LDL, VLDL and free fatty acids in the plasma. The specification clearly describes the reduction of blood glucose, triglyceride levels, total cholesterol, LDL, VLDL and free fatty acids following *in vivo* administration of the claimed compounds to animal models of diabetes and hypercholesterolemia. (*See, e.g.*, page 92, line 1 to page 96, line 9.) Thus, the specification enables one skilled in the art to carry out the claimed method, and reconsideration of this basis for rejection is respectfully requested. Applicants submit that newly added claims 88-93, directed to a method for the treatment or prevention of conditions associated with high blood glucose, high triglycerides and/or high total cholesterol, are also enabled for at least the same reasons, and no rejection under 35 U.S.C. § 112, first paragraph should be forthcoming.

With respect to claims 27, 31, 68, 72, 80 and 83, these claims have been amended to limit the method to treating or preventing diabetes or impaired glucose tolerance. The Examiner has indicated that the treatment of diabetes is enabled by the specification. Applicants submit that the prevention of diabetes is also enabled by the specification. One of skill in the art at the time the instant specification was filed recognized that medications that lower blood glucose levels could lower the risk of progression from impaired glucose tolerance to type II diabetes. *See, e.g.*, Pickavance *et al.*, *Br. J. Pharmacol.* 125:767 (1998); Chaisson *et al.*, *Diabetes Care* 21:1720 (1998), copies of which are herewith provided for the benefit of the Examiner. Thus, the specification enables one skilled in the art to carry out the claimed method, and reconsideration of this basis for rejection is respectfully requested.

With respect to claims 29, 33, 70, 74 and 85, these claims are limited to a method for the treatment or prophylaxis of disorders related to Syndrome X. Chaisson *et al.*, discussed above, notes that glucose intolerance is part of a clustering of risk factors for cardiovascular disease, obesity, hypertension, high triglyceride levels and low HDL cholesterol, termed Syndrome X. As discussed above, the specification clearly describes the reduction of blood glucose, triglyceride levels, total cholesterol, LDL, VLDL and free fatty acids levels following *in vivo* administration of the claimed compounds to animal models of diabetes and hypercholesterolemia. (*See* page 92, line 1 to page 96, line 9.) In

43

addition, WO 97/25042, cited in the instant specification, discloses propanoic acid derivatives for the treatment of syndrome X having PPARα and/or PPARγ agonist activity which reduced blood glucose, triglyceride and fatty acid levels following *in vivo* administration to diabetic mice. Thus, the specification enables one skilled in the art to carry out the claimed method, and reconsideration of this basis for rejection is respectfully requested. Applicants submit that newly added claim 87, also directed to a method for the treatment or prophylaxis of disorders related to Syndrome X, is enabled for at least the same reasons, and no rejection under 35 U.S.C. § 112, first paragraph should be forthcoming.

Newly added claims 94-99 are directed to a method for the treatment or prevention of hyperlipidemia, hypercholesterolemia, hyperglycemia, insulin resistance, psoriasis, obesity, leptin resistance and/or type II diabetes. Applicants submit that the specification enables the treatment or prevention of each of the recited diseases or disorders. Hyperlipidemia, hypercholesterolemia, hyperglycemia, insulin resistance and type II diabetes have been discussed above. Regarding obesity and leptin resistance, the specification describes materials showing a link between leptin resistance, obesity, impaired glucose tolerance and diabetes. (*See* page 5, lines 4-12.) Furthermore, as discussed above, the specification clearly describes the reduction of blood glucose, triglyceride levels, total cholesterol, LDL, VLDL and free fatty acids levels following *in vivo* administration of the claimed compounds to animal models of diabetes and hypercholesterolemia. (*See* page 92, line 1 to page 96, line 9.) These animal models included the db/db mouse, which is obese, hyperglycemic, hyperinsulinemic and insulin resistant, the ob/ob mouse, which is obese, leptin resistant, hyperphagic, hyperglycemic and hyperinsulinemic, and the fa/fa rat, which is obese, leptin resistant, hyperphagic and hyperlipidemic. In addition, Pickavance *et al.*, discussed above, describes treatment of pre-diabetic Zucker Diabetic Fatty (ZDF) rats, which are obese, leptin resistant and hyperphagic, with a PPAR agonist, which prevented progression of impaired glucose tolerance to diabetes. Thus, the specification enables one skilled in the art to carry out the claimed method, and no rejection under 35 U.S.C. § 112, first paragraph should be forthcoming.

44

Newly added claims 100-108 are directed to methods of activating PPARα and/or PPARγ in a cell, while newly added claims 109-111 are directed to a method for the treatment and/or prevention of a condition mediated by PPARα and/or PPARγ. Applicants submit that the *in vitro* data provided at page 90, line 24 to page 91, line 17, and the *in vivo* data provided at page 92, line 1 to page 96, line 9, enables one skilled in the art to carry out the claimed methods, and no rejection under 35 U.S.C. § 112, first paragraph should be forthcoming.

CONCLUSION

It is believed that claims 1-3, 5-13, 24-27, 29-31, 33, 34 and 65-68, 70-72, 74, 75, 77-80, 82, 83 and 85-111 are now in condition for allowance, early notice of which would be appreciated. If any outstanding issues remain, the Examiner is invited to telephone the undersigned at the number indicated below to discuss the same. No fees are believed due at this time other than the additional claim fee. If, however, any other fees are due, the Commissioner is authorized to charge any such fee to our Deposit Account No. 50-3221.

Respectfully submitted,

Dated: November 17, 2006

Milagros A. Cepeda
Attorney for Applicants
Reg. No. 33,365

Dr. Reddy's Laboratories, Inc.
200 Somerset Corporate Blvd.
Seventh Floor
Bridgewater, NJ 08807-2862
Tel. No.: 908-203-6504
Fax No.: 908-203-6515

600612

45

The STOP-NIDDM Trial

An international study on the efficacy of an α-glucosidase inhibitor to prevent type 2 diabetes in a population with impaired glucose tolerance: rationale, design, and preliminary screening data

JEAN-LOUIS CHIASSON, MD
RAMON GOMIS, MD
MARKOLF HANEFELD, DSC
ROBERT G. JOSSE, MBBS

AVRAHAM KARASIK, MD
MARKKU LAAKSO, MD
THE STOP-NIDDM TRIAL RESEARCH
GROUP

OBJECTIVE— To describe the rationale and design, and to discuss the preliminary screening data, of the Study to Prevent NIDDM (STOP-NIDDM Trial), an international study on the efficacy of the α-glucosidase inhibitor acarbose in preventing or delaying the development of type 2 diabetes in a population with impaired glucose tolerance (IGT).

RESEARCH DESIGN AND METHODS— A total of 1,418 subjects diagnosed with IGT according to the World Health Organization's criteria and having a fasting plasma glucose concentration ≥5.6 mmol/l were randomized in a double-blind fashion to receive either acarbose (100 mg t.i.d.) or placebo for a predictive median follow-up period of 3.9 years. The primary outcome is the development of type 2 diabetes diagnosed using a 75-g oral glucose tolerance test according to the new criteria. The secondary outcomes are changes in blood pressure, lipid profile, insulin sensitivity, cardiovascular events, and morphometric profile.

RESULTS— Screening was performed in a high-risk population. As of 1 March 1997, 4,424 subjects had been screened, and data were available for 3,919 (88.5%) subjects. Of these subjects, 1,200 (30.6%) had glucose intolerance. Of the subjects with glucose intolerance, 521 (13.3%) had previously undetected type 2 diabetes, and 679 (17.3%) had IGT. Of the IGT population, 412 (60.7%) subjects were eligible for the study. This population had the following characteristics: the mean age was 54.8 years, 52% of the subjects were female, 53% had more than one risk factor for type 2 diabetes, >90% had a family history of diabetes, 78.2% had a BMI ≥27 kg/m², 47.5% had high blood pressure, 51.2% had dyslipidemia, and 22.8% of the women had a history of gestational diabetes.

CONCLUSIONS— Screening of a high-risk population yields one eligible subject per every 10 volunteers screened. This study should definitely answer the question of whether acarbose can prevent or delay the progression of IGT to type 2 diabetes mellitus.

Diabetes Care 21:1720–1725, 1998

Type 2 diabetes is well recognized as a major health problem associated with increased morbidity and mortality and high health care costs (1,2). It is believed that all type 2 diabetic patients must pass through a phase of impaired glucose tolerance (IGT) before developing diabetes. IGT was first recognized as an entity in 1979 by the National Diabetes Data Group (3) and the World Health Organization (WHO) Expert Committee on Diabetes (4). Recently, the diagnostic criteria have been refined further by the Expert Committee on the Diagnosis and Classification of Diabetes Mellitus (5).

The prevalence of IGT among adults varies between 3 and 10% in European populations and 11 and 20% in North American populations (6). Although IGT is not associated with diabetes-specific complications, it does increase the risk of developing type 2 diabetes and cardiovascular disease (7,8). Numerous studies have shown that the progression of IGT to type 2 diabetes can vary between 1.5 and 7.3% per year in different ethnic groups and in various regions of the world (7). Furthermore, glucose intolerance is part of a clustering of risk factors for cardiovascular disease, which includes central obesity, hypertension, high triglyceride (TG) levels, and low HDL cholesterol and has been termed syndrome X, or the metabolic syndrome (9). These disorders are all characterized by insulin resistance and hyperinsulinemia.

Insulin resistance is generally believed to be the first abnormality leading to glucose intolerance (10). However, as long as the β-cell can compensate by increasing insulin secretion, normal glucose tolerance is maintained. Only when the β-cell fails to compensate does IGT develop, resulting initially in postprandial hyperglycemia (10). Such hyperglycemia further exacerbates the insulin resistance and the β-cell defect, a phenomenon known as glucose toxicity (11). A large proportion of these IGT subjects eventually progress to development of type 2 diabetes (12).

With this understanding, it is hypothesized that any therapeutic intervention in

IGT subjects that could decrease insulin resistance or protect the β-cell, or both, could prevent or delay the progression of IGT to type 2 diabetes. Acarbose, an α-glucosidase inhibitor, qualifies as a potential pharmacological agent for the prevention of type 2 diabetes. In a pilot study, we have shown in IGT subjects that acarbose, by delaying the absorption of carbohydrate by the gut, decreases the postprandial increase in plasma glucose, resulting in lower postprandial plasma insulin levels (13). This decrease in plasma insulin releases the strain on the β-cell and, theoretically, should protect the insulin-producing cells. We have also shown in the same subjects that 4 months of treatment with acarbose resulted in a significant decrease in insulin resistance (13). Furthermore, we have demonstrated that the long-term use of acarbose in the treatment of type 2 diabetic subjects is not associated with toxicity (14). Acarbose may therefore be a drug of choice for the long-term prevention of diabetes.

These observations provided the rationale for the Study to Prevent NIDDM (STOP-NIDDM Trial). Its primary objective is to evaluate in IGT subjects the effect of acarbose on the conversion rate of IGT to type 2 diabetes. Secondary objectives are to evaluate the effects of acarbose treatment on glucose tolerance, insulin sensitivity, hyperinsulinemia, anthropometric measurements, blood pressure, lipid profile, and the appearance and progression of cardiovascular events. In this article, we describe the study design of the STOP-NIDDM Trial and discuss the preliminary screening data.

RESEARCH DESIGN AND METHODS— The STOP-NIDDM Trial is an international multicenter placebo-controlled randomized study being conducted in Canada, Germany, Austria, the Nordic countries (Norway, Denmark, Sweden, and Finland), Israel, and Spain.

Study population
Subjects were recruited from a high-risk population mainly through newspaper advertisement. Subjects enrolled in the study were all diagnosed with IGT based on the 2-h plasma glucose post–75-g glucose test according to the WHO criteria (plasma glucose ≥7.8 mmol/l [≥140 mg/dl] and <11.1 mmol/l [<200 mg/dl]). Furthermore, they all had fasting plasma glucose (FPG) ≥5.6 but <7.8 mmol/l (≥101 but <140 mg/dl), which has been shown to increase the risk of progression to type 2 diabetes 3.4-fold (15).

In the new diagnostic criteria for diabetes, the cutoff for FPG has been lowered to 7.0 mmol/l (126 mg/dl). As a result, subjects are considered diabetic based on an FPG measurement between 7.0 and 7.8 mmol/l (126 and 140 mg/dl). Nevertheless, the 2-h plasma glucose post–75-g glucose load remained within the original (and current) diagnostic level for IGT (7.8–11.0 mmol/l [140–199 mg/dl]). Men and women between the ages of 40 and 70 years and having a BMI between 25 and 40 kg/m² were recruited. Subjects were excluded if they had a serum creatinine level ≥130 μmol/l (≥1.5 mg/dl), a fasting serum TG level ≥10 mmol/l (≥886 mg/dl), liver enzymes elevated to ≥1.8 times the upper limit of normal (ULN), or a TSH ≥1.5 times above the ULN or below the lower limit of normal (<0.3 mU/l). Subjects who had been treated within the last 3 months with systemic glucocorticoids, β-blockers, thiazide diuretics, and nicotinic acid were also excluded. Key exclusion criteria were based on the use of drugs that were likely to be associated with abnormal intestinal motility or altered absorption of nutrients. All subjects with a recent cardiovascular event were also excluded.

Study design
Altogether, 1,418 subjects with IGT and an FPG concentration ≥5.6 but <7.8 mmol/l (≥101 but <140 mg/dl) were randomized in a double-blind fashion to treatment with either placebo or acarbose, which was taken with the first bite of each meal three times a day. At entry, all subjects were given instruction in following a weight-reducing or weight-maintaining diet and were encouraged to exercise regularly. To avoid or minimize the gastrointestinal side effects of acarbose, its administration was started at 50 mg/day and titrated gradually (50 mg/day per 2 weeks) to a maximum of 100 mg t.i.d. or the maximum tolerated dosage. The subjects are examined every 3 months by the coordinating nurse and every 6 months by the investigator for a median follow-up time of 3.9 years. At the end of the treatment period, there will be a 3-month single-blind placebo washout period, after which the outcome measures will be repeated. The protocol has been approved by appropriate institutional review boards, and each subject has signed an informed consent form.

Outcome measures
The primary outcome is the development of diabetes based on a 75-g oral glucose tol-

erance test (OGTT) (5). The OGTT is performed yearly; however, at the visits occurring every 3 months, subjects are scheduled for an OGTT if their FPG level is ≥7.0 mmol/l (126 mg/dl). If the OGTT confirms the diagnosis of type 2 diabetes, the subjects will have reached the primary end point. However, they will be kept in the study, will continue to receive the study medication in a blinded fashion, and will be monitored for the secondary end points.

Among the secondary objectives is the assessment of any improvement in glucose tolerance (i.e., reversal of IGT to normal glucose tolerance, based on the OGTT) that could result from acarbose treatment. During the OGTT, samples will also be drawn for plasma insulin measurement to evaluate the effect of the treatment on baseline insulin and its response to a glucose challenge. Before randomization, all subjects fulfilling the enrollment criteria had a second OGTT to measure basal and stimulated insulin response. The investigators are blinded to the results of this second test, which will be included, however, in the final analysis. Insulin sensitivity is measured using the frequently sampled intravenous glucose tolerance test (FSIGTT) minimal model (16) in 10% (n = 120) of the study population, randomly selected. The insulin sensitivity index (S_i) and glucose effectiveness (S_G) will be quantified by application of Bergman's minimal model to an insulin-modified FSIGTT (17). The insulin and glucose data are then submitted by Diane Finegood (Burnaby, British Columbia, Canada) to the MINMOD computer system program for the determination of S_i and S_G.

Anthropometric measurements are made with the participant wearing an examining gown after having removed shoes and upper garments. A tape measure is used to measure waist circumference at the level of the iliac crest, and hip circumference at the level of the largest circumference or the greater trochanters. The waist-to-hip ratio is used as an index of upper-body versus lower-body adiposity. BMI is calculated as weight (in kilograms) divided by height (in meters) squared.

All cardiovascular events are documented and confirmed by appropriate clinical and/or laboratory data: 1) myocardial infarction: electrocardiogram (ECG) abnormalities and elevation of enzymes; 2) cerebrovascular accident: abnormal findings on neurological examination; 3) congestive heart failure: chest X-ray abnormalities. All subjects undergo a standard digital 12-lead

ECG before randomization, at the end of the study, and whenever clinically indicated. All ECGs obtained during the study are read by two independent cardiologists for cross-validation: Martin Green (Ottawa, Ontario, Canada) and Wolfgang Rafflenbeul (Hanover, Germany). Blood pressure is monitored every 3 months according to the recommendations of the Canadian Hypertension Society (18,19). The blood pressure is measured after subjects rest for at least 5 min, and three blood pressure readings are obtained at each visit. The average of the three readings is used, and blood pressure is considered elevated if the average is >140/90 mmHg. If elevated, blood pressure is remeasured within 2 weeks, and if hypertension is confirmed, antihypertensive treatment is started. The first drug used is a calcium channel blocker. Only when calcium channel blockers are not sufficient to control blood pressure are α-blockers or ACE inhibitors added, because these agents have been shown to improve insulin sensitivity (20). The β-blockers and thiazides are used only as a last resort because they are known to decrease insulin sensitivity.

Nutritional evaluations are performed before randomization and once a year thereafter. Evaluations are made using a 3-day dietary diary in which all food items and quantities as well as physical activities are documented for three representative days (two weekdays and one weekend day). From the beginning of the study, all subjects are given instruction on how to follow a weight-reducing diet if they are overweight or a weight-maintaining diet if they are of normal weight. The nutritional journals are used to determine any major change in the quantity or quality of the diet over the study period.

HbA$_{1c}$ is measured by high-performance liquid chromatography (21) at baseline, every year after randomization, and at the end of the study. Serum insulin is measured using a highly specific two-site monoclonal antibody immunoradiometric assay (22). Serum total cholesterol (TC) and TG levels are measured enzymatically (23). HDL cholesterol is measured as cholesterol after precipitation of non-HDL cholesterol using dextran sulfate–magnesium chloride (23). LDL cholesterol is then calculated for samples in which TG concentration is <4.51 mmol/l, using the following formula (24): LDL = [(TC − HDL) − TG] ÷ 2.2. ∴∴ The lipid profile is also performed at baseline, every year, and at the end of the washout period. Plasma glucose is meas-

ured by the glucose oxidase or hexokinase method.

Laboratories and cross-validation

One of the investigators (T.M.S. Wolever, Toronto, Ontario, Canada), is responsible for the cross-validation of data. Pooled frozen human serum or plasma samples are sent to each of the participant laboratories at 4-month intervals for glucose, insulin, and lipid analysis. For HbA$_{1c}$, fresh whole blood samples are sent each time. Each sample is analyzed 10 times by each laboratory.

Glucose. Between- and within-laboratory means, SDs, and coefficients of variation (CVs) are calculated as measures of variability. Repeated measures two-way analysis of variance (ANOVA) will be performed, with the variables being analyte concentration and laboratory to determine whether differences between means from the different centers are significant.

For each concentration of each analyte, each laboratory's results will be displayed as the SD index (SDI), calculated as follows: SDI = (Lab mean − Group mean) ÷ Group SD.

An SDI >2 indicates that the laboratory result is >2 SD from the mean and is not in agreement with the group. Each laboratory's result for each concentration will be plotted on a Levey-Jennings control chart where the x-axis is the run number (throughout the study, each sample of pooled serum will be run 10 times) and the y-axis is the SDI.

A laboratory can be considered to differ consistently from the group mean if its SDI is >2 on two consecutive occasions. The group mean for the different concentrations of the affected analyte will be plotted against the laboratory mean, and if, by inspection, the relationship is linear, a regression equation will be calculated by least-squares method to allow correction of the laboratory's results to the group mean as follows: $y = mx + b$, where y is the group mean, x is the individual laboratory's result, m is the slope, and b is the y-intercept. If the discrepancy is not linear, a curve will be fit.

Insulin and lipids. For each concentration, the between- and within-laboratory means, SDs, and CVs will be presented, and repeated measures ANOVA performed, to determine whether there are significant differences between laboratories. Regression analysis will be performed for the different concentrations of each analyte, and each central laboratory will receive graphs on which are plotted its results against those of the other laboratories. In addition, a graph will be plot-

ted to show the relationship of all laboratories to the group mean (i.e., group mean on the y-axis) and regression equations will be given to adjust all results to the group mean.

HbA$_{1c}$. Within-laboratory variation will be assessed from the difference of the duplicate samples. Between-laboratory variation will be assessed by repeated measures ANOVA and regression analysis and will be displayed as for insulin and lipids.

Sample size estimation

Calculation of sample size was done using a two-tailed α of 0.05 and 1-β of 90% assuming *1*) a conversion rate of 7% per year, *2*) a 36% risk reduction (from 27.1% in the placebo group to 17.2% in the acarbose-treated group), and *3*) a 10% dropout rate. It was calculated that 600 subjects needed to be randomized per treatment group.

Statistical analysis

The intent-to-treat analysis will be the primary analysis for this study. Only protocol-compliant subjects will be included in the secondary analysis. The primary variable for the study is time to development of diabetes. The data will be displayed using Kaplan-Meier curves for the two treatment groups. Formal analysis will be done using a Cox proportional hazards model including covariates such as treatment of any baseline variable that may influence outcome. The secondary end points will be summarized using proportions and analyzed using repeated measures ANOVA.

Interim analysis

An O'Brien-Fleming type of boundary (25) will be used for the primary end point, that is, the time to the development of diabetes. The first analysis will be done after randomized subjects have been followed for a minimum of 1 year. Subsequent analysis will be done at ~6-month intervals for a maximum of five such analyses (including the first analysis at the 1-year follow-up). The results will be considered significant at P values of 0.00001, 0.001, 0.008, 0.023, and 0.043 for the first through final analysis, respectively.

The Data Safety and Quality Review Committee

An independent committee, called the Data Safety and Quality Review Committee (DSQRC), has been created to review the results of data analysis at various intervals throughout the study without unblinding the investigators and the sponsor and to guarantee scientific integrity and credibility.

The DSQRC is composed of a chairperson (Charles M. Clark, Jr., Indianapolis, IN), a biostatistician (Wayne Taylor, Hamilton, Ontario, Canada), and two clinical epidemiologists (Bryan Haynes, Hamilton, Ontario, Canada, and Lothar Heinemen, Berlin, Germany). The committee will have the mandate to assess at various intervals *1)* the quality of the data; *2)* the efficacy of the study medication; and *3)* the safety of the study medication. The DSQRC reports to the steering committee, which is composed of the principal investigator (J.-L.C.); five coinvestigators, R.G. (Spain), M.H. (Germany), R.G.J. (Canada), A.K. (Israel), and M.L. (Finland); and five representatives from our sponsor, Bayer.

RESULTS— Screening for the STOP-NIDDM Trial was initiated early in 1996, and final enrollment was completed on 28 February 1998. The subjects were screened from a high-risk population and were recruited mainly through newspaper advertisement. In this article, we present the screening data up to 1 March 1997. At that date, 4,424 subjects had undergone an OGTT. Data were available on 88.5% (*n* = 3,919) of the subjects (Table 1). Of the overall population screened, 30.6% (*n* = 1,200) had glucose intolerance. Of the latter group, 13.3% (*n* = 521) had previously undetected diabetes, 17.3% (*n* = 679) had IGT; of the IGT subjects, 10.5% (*n* = 412) had an FPG ≥5.6 mmol/l and were eligible for the study. Reviewing the data from this study population, 7% had an FPG ≥7.0 but <7.8 mmol/l (≥126 but <140 mg/dl) on one occasion. The new diagnostic criteria require two FPG levels ≥7.0 mmol/l to establish the diagnosis of diabetes.

The age of the population screened did not differ among various groups, but a small upward trend was observed, with increasing glucose intolerance from 52.5 years of age for those with a normal OGTT result to 54.9 years of age for those with type 2 diabetes.

At least one known risk factor for the development of diabetes was identified in >90% of the subjects. However, the number of risk factors did not discriminate among the subjects who had diabetes, those who had IGT with an FPG level above or below 5.6 mmol/l (101 mg/dl), and those who had normal glucose tolerance. This finding is not unexpected, because recruitment of the screened population was based on risk factors. For the same reason, the family history of diabetes, which was present in

Table 1—*Characteristics of the screened population*

| Characteristics | Glucose tolerance | | | |
	Normal	Total IGT	IGT + FPG ≥5.6 mmol/l	Diabetes
OGTT results (%)	69.5	17.3	10.5	13.3
Mean age (years)	52.5	54.4	54.8	54.9
Diabetes history (%)	91.0	93.0	93.0	94.0
BMI >27 kg/m² (%)	64.5	76.3	78.2	74.3
Hypertension history (%)	34.7	43.8	47.5	50.7
Dyslipidemia history (%)	44.2	49.8	51.2	59.3
Gestational diabetes history (% of women)	26.0	29.7	22.8	28.0

n = 3,919.

>90% of the subjects screened, did not discriminate among the groups (i.e., normal versus IGT versus diabetes). Obesity defined as BMI >27 kg/m² tended to be less frequent in the normal population screened (64.5%) compared with those with glucose intolerance (76.3%). Known history of high blood pressure and dyslipidemia tended to increase with worsening of glucose tolerance. The presence of hypertension was only 34.7% in those with normal glucose tolerance, compared with 43.8% in those with total IGT, 47.5% in those with IGT + FPG ≥5.6 mmol/l, and 50.7% in those with diabetes. Similarly, history of known dyslipidemia was lower in those with normal OGTT (44.2%) than in those with IGT (49.8%), IGT + FPG ≥5.6 mmol/l (51.2%), or diabetes (59.3%). Of the women, >27.0% (*n* = 237) had a history of gestational diabetes, but this prevalence did not differ between those with glucose intolerance and those with normal OGTT results.

CONCLUSIONS— The STOP-NIDDM Trial Research Group has invested much effort, thought, and discussion in developing the study design, deciding on the sample size, and choosing an appropriate treatment for the prevention of type 2 diabetes. The preliminary screening data confirm the feasibility of the STOP-NIDDM Trial and provide interesting information on the population under study.

The design is a straightforward randomized double-blind placebo-controlled study. Because it is a multicenter and multinational trial, it is particularly complex to coordinate, and monitoring of the various sites involved is especially difficult. Fortunately, our sponsor, Bayer, has provided the infrastructure and the qualified personnel to ensure that the study is well coor-

dinated and that the monitoring of the various sites is adequate and timely. However, to guarantee quality and credibility of the data, randomization and analysis of the data are done by an independent group. The data are then routed to the DSQRC, which is independent of both the sponsor and the research group. The multinational character of the study also makes it difficult to have all the laboratory parameters measured in one central laboratory. For this reason, a cross-validation protocol has been elaborated to ensure reproducibility of the various assays.

In deciding on the duration of a trial in which we were monitoring subjects for the appearance of diabetes, we had to consider the expected number of events per year and the number of subjects studied; we decided to do a 3-year study, to begin after the last subject was recruited, with a predictive median follow-up of 3.9 years. According to the literature, the conversion rate of IGT to type 2 diabetes varies from 1.5 to 7.3%, with a mean of ~3–4% per year (7). The most consistent predicting factor for the progression to diabetes is the FPG (15). Using an FPG ≥5.6 mmol/l (≥101 mg/dl) in the inclusion criteria increases the risk of conversion to diabetes by 3.4-fold (15). Therefore, in our IGT population, the expected conversion rate should be ~10% per year. To be conservative, we have used a conversion rate of 7% in our estimation of the sample size. We have also assumed that a risk reduction of 36% would be clinically meaningful, and more important, cost-effective. Eastman et al. (26) have suggested that a 20% reduction in the risk of developing diabetes would have a cost-effectiveness of $42,135 per quality-adjusted life years. Furthermore, the Da Qing study (27) showed that

changes in lifestyle such as diet and exercise decreased the conversion rate by ~40%. It was therefore decided that a 36% reduction in risk was realistic. Finally, we have assumed a 10% dropout rate for the 3-year study period. This rate is based on the experience of the Early Diabetes International Trial (EDIT), a 3-year multicenter study on the efficacy of acarbose, metformin, and acarbose plus metformin in preventing the deterioration of glucose tolerance (R. Holman, personal communication). With these conservative assumptions, the estimated population size required to meet the primary objective was 1,200 subjects randomized equally into each treatment for a 3-year period. To increase the power, it was decided to increase the number of subjects to 1,400 and to prolong the treatment until the last subject has been on the study medication for 3 years, which gives us a predictive median treatment period of 3.9 years.

The choice of treatment was based on our understanding of the pathophysiology of glucose intolerance and on our experience with the drug acarbose. We had already shown in IGT subjects that acarbose was effective in releasing the strain on the β-cell, thus theoretically protecting the β-cells, and in decreasing insulin resistance (13). Furthermore, we had shown in a long-term study of patients with type 2 diabetes (14) that acarbose was nontoxic and well tolerated. Thus, it appeared to have all the required characteristics of a suitable drug for the long-term prevention of type 2 diabetes.

The preliminary screening data offer some insights into the high-risk population under study. The data show that 10.5% (1 in 10) of subjects screened were eligible for the study. Even when one considers that 17.3% had IGT, this percentage is still lower than one would expect in a high-risk population. The prevalence of IGT in European and North American adult populations is already close to 10% (6). However, most of the IGT subjects are >65 years of age; the mean age of our screened population is 52.7 ± 0.01 years. This finding suggests that older subjects may have been less interested in participating in the study, most likely because they believed that they were too old to benefit from such a prevention program. The younger population seemed to be more concerned about their genetic background and their immediate future health status. Our screened population contained slightly more women than men, but the sex distribution was not significantly

different from data on glucose-intolerant subjects found in the literature (6).

The known family history of diabetes did not discriminate among those with IGT, those with type 2 diabetes, and those with normal OGTT results. This outcome was not unexpected, given that family history was the most common reason for responding to our advertisement. The fact that there was less obesity among subjects with normal glucose tolerance than among those with glucose intolerance is consistent with the role of obesity in the development of IGT and diabetes (28). The increased prevalence of known history of hypertension and dyslipidemia among those with increasing glucose intolerance suggests that increasing hyperglycemia may contribute to the development of some of the risk factors for cardiovascular disease. This association has also been suggested by the San Luis Valley Diabetes Study (29). This does not rule out a direct effect of hyperglycemia, per se, as a risk factor for cardiovascular disease (30,31).

The STOP-NIDDM Trial should be completed by the year 2001. It has been carefully designed to assess the effectiveness of acarbose in preventing the conversion of IGT to diabetes. Screening from a high-risk population has confirmed the feasibility of recruitment for such a study. We believe that the results could have a major impact on the development of diabetes, and we are hopeful that they will provide evidence that specific intervention can successfully reduce the overall rate of diabetes.

APPENDIX

The STOP-NIDDM Trial Research Group

Canada: S. Arndt, H. Conter, G.A. Costain, D. Finegood, H.C. Gerstein, J.A. Hunt, C.J. Joyce, D. Lau, L.A. Leiter, S.M. Ludwig, P. Maheux, N.W. Rodger, S.A. Ross, E.A. Ryan, T.M.S. Wolever, J.-F. Yale. Germany: U.R. Folsch, H.-J. Lüddeke, C. May, S. Mattaei, H. Schmechel, G. Schernthaner (Austria), E. Standl, J. Ziegelasch. Israel: H. Bar On, Y. Kantor, M. Maislos, M. Rapoport, I. Raz, P. Segal. Scandinavia: D. Anderson, S.

Efendic, J. Eriksson, B. Forsen, K. Hermansen, S. Vaaler. Spain: I. Conget. Bayer: I. Elias (Canada), H. Fischer (Germany).

References
1. Wetterhall SF, Olson DR, DeStefano F, Stevenson JM, Ford ES, German RR, Will JC, Newman JM, Sepe SJ, Vinicor F: Trends in diabetes and diabetic complications, 1980–1987. *Diabetes Care* 15:960–967, 1992
2. Rubin RJ, Altman WM, Mendelson DN: Health care expenditures for people with diabetes mellitus, 1992. *J Clin Endocrinol Metab* 78:809A–809F, 1994
3. National Diabetes Data Group: Classification and diagnosis of diabetes mellitus and other categories of glucose intolerance. *Diabetes* 28:1039–1057, 1979
4. World Health Organization: *Prevention of Diabetes Mellitus: Report of a WHO Study Group.* Geneva, World Health Org., 1994 (Tech. Rep. Ser., no. 844)
5. The Expert Committee on the Diagnosis and Classification of Diabetes Mellitus: Report of the Expert Committee on the Diagnosis and Classification of Diabetes Mellitus. *Diabetes Care* 20:1183–1197, 1997
6. King H, Rewers M, WHO Ad Hoc Diabetes Reporting Group: Global estimates for prevalence of diabetes mellitus and impaired glucose tolerance in adults. *Diabetes Care* 16:157–177, 1993
7. Harris MI: Impaired glucose tolerance: prevalence and conversion to NIDDM. *Diabet Med* 13 (Suppl. 2):S9–S11, 1996
8. Donahue RP, Orchard TJ: Diabetes mellitus and macrovascular complications: an epidemiological perspective. *Diabetes Care* 15:1141–1155, 1992
9. Reaven GM: Role of insulin resistance in human disease. *Diabetes* 37:1595–1607, 1988
10. DeFronzo RA, Bonadonna RC, Ferrannini E: Pathogenesis of NIDDM: a balanced overview. *Diabetes Care* 15:318–368, 1992
11. Rossetti L, Giaccari A, DeFronzo RA: Glucose toxicity. *Diabetes Care* 13:610–630, 1990
12. Dowse GK, Zimmet PZ, King H: Relationship between prevalence of impaired glucose tolerance and NIDDM in a population. *Diabetes Care* 14:968–974, 1991
13. Chiasson J-L, Josse RG, Leiter LA, Mihic M, Nathan DM, Palmason C, Cohen RM, Wolever TMS: The effect of acarbose on insulin sensitivity in subjects with impaired glucose tolerance. *Diabetes Care* 19:1190–1193, 1996
14. Chiasson J-L, Josse RG, Hunt JA, Palmason C, Rodger NW, Ross SA, Ryan EA, Tan MH, Wolever TMS: The efficacy of acarbose in the treatment of patients with non-insulindependent diabetes mellitus: a multicenter controlled clinical trial. *Ann Intern Med* 121:928–935, 1994

15. Saad MF, Knowler WC, Pettitt DJ, Nelson RG, Mott DM, Bennett PH: The natural history of impaired glucose tolerance in the Pima Indians. *N Engl J Med* 319:1500–1506, 1988

16. Bergman RN, Prager R, Volund A, Olefsky JM: Equivalence of the insulin sensitivity index in man derived by the minimal model method and the euglycemic glucose clamp. *J Clin Invest* 79:790–800, 1987

17. Finegood DT, Hramiak IM, Dupré J: A modified protocol for estimation of insulin sensitivity with the minimal model of glucose kinetics in patients with insulin-dependent diabetes. *J Clin Endocrinol Metab* 70:1538–1549, 1990

18. Dawson KG, McKenzie JK, Ross SA, Chiasson J-L, Hamet P: Report of the Canadian Hypertension Society Consensus Conference: 5. Hypertension and diabetes. *CMAJ* 149:821–826, 1993

19. Carruthers SG, Larochelle P, Haynes RB, Petrasovits A, Schiffrin EL: Report of the Canadian Hypertension Society Consensus Conference: 1. Introduction. *CMAJ* 149:289–293, 1993

20. Chan JCN, Yeung VTF, Leung DHY, Tomlinson B, Nicholls MG, Cockram CS: The effects of enalopril and nifedipine on carbohydrate and lipid metabolism in NIDDM. *Diabetes Care* 17:859–862, 1994

21. Cole RA, Soeldner JS, Dunn PJ, Bunn HF: A rapid method for the determination of glycosylated hemoglobins using high pressure liquid chromatography. *Metabolism* 27:289–301, 1978

22. Sobey WJ, Beer SF, Carrington CA, Clark PMS, Frank BH, Gray IP, Luzio SD, Owens DR, Schneider AE, Siddle K, Temple RC, Hales CN: Sensitive and specific two-site immunoradiometric assays to human insulin, proinsulin, 65-66 split and 32-33 split proinsulins. *Biochem J* 260:535–541, 1989

23. Warnick GR, Bendersen J, Albers JJ: Dextran sulfate-Mg^{2+} precipitation procedure for quantitation of high-density-lipoprotein cholesterol. *Clin Chem* 28:1379–1388, 1982

24. Friedewald WT, Levy RI, Fredrickson DS: Estimation of concentration of low-density lipoprotein cholesterol in plasma, without the use of the preparative ultracentrifuge. *Clin Chem* 18:499–502, 1972

25. DeMetz DL, Lan KKG: Interim analysis: the alpha spending function approach. *Stat Med* 13:1341–1359, 1994

26. Eastman RC, Javitt JC, Herman WH, Dasbach EJ, Harris MI: Prevention strategies for non-insulin-dependent diabetes mellitus: an economic perspective. In *Diabetes Melli -* tus LeRoith D, Taylor SI, Olefsky JM, Eds. Philadelphia, Lippincott-Raven, 1996, p. 621–630

27. Pan X, Li G, Hu Y-H, Wang J, Yang W, Zuo A, Ze H, Lin J, Xiao J-Z, Cao H, Liu P-A, Jiang X, Jiang Y, Wang J, Zheng H, Zhang H, Bennett PH, Howard BV: Effects of diet and exercise in preventing NIDDM in people with impaired glucose tolerance: the Da Qing IGT and Diabetes Study. *Diabetes Care* 20:537–544, 1997

28. Mykkänen L, Laakso M, Uusitupa M, Pyorala K: Prevalence of diabetes and impaired glucose tolerance in elderly subjects and their association with obesity and family history of diabetes. *Diabetes Care* 13:1099–1105, 1990

29. Burchfiel CM, Hamman RF, Marshall JA, Baxter J, Kahn LB, Amirani JJ: Cardiovascular risk factors and impaired glucose tolerance: the San Luis Valley Diabetes Study. *Am J Epidemiol* 31:57–70, 1990

30. Singer DE, Nathan DM, Anderson KM, Wilson PW, Evans JC: Association of HbA$_{1c}$ with prevalent cardiovascular disease in the original cohort of the Framingham Heart Study. *Diabetes* 41:202–208, 1992

31. Gerstein HC, Yusuf S: Dysglycaemia and risk of cardiovascular disease. *Lancet* 347:949–950, 1996

The development of overt diabetes in young Zucker Diabetic Fatty (ZDF) rats and the effects of chronic MCC-555 treatment

[1,3]L. Pickavance, [1]P.S. Widdowson, [1]P. King, [2]S. Ishii, [2]H. Tanaka & [1]G. Williams

[1]Diabetes and Endocrinology Research Group, Department of Medicine, University of Liverpool, Liverpool and [2]Mitsubishi Chemical Corporation, Yokohama Research Center, Yokohama, Japan

1   Young (6-week-old) pre-diabetic Zucker Diabetic Fatty (ZDF) rats displaying impaired glucose tolerance (IGT), moderate hyperglycaemia and hyperinsulinaemia were treated with the novel thiazolidinedione, MCC-555, for 28 days, during which time β-cell failure and progression to overt diabetes occurs.

2   Treated ZDF rats exhibited consistently lower blood glucose levels than vehicle-treated diabetic controls, with a delayed rise and lower plateau levels. MCC-555 maintained plasma insulin levels throughout the treatment period, whereas these fell by 40% in untreated ZDF rats.

3   The rise in body weight was maintained in MCC-555-treated rats, whereas vehicle-treated rats exhibited blunted body weight gain after 8 weeks of age. Daily food intake was higher in diabetic, as compared to non-diabetic rats, but treatment did not modify food intake in diabetic rats. Water intake was lower in treated ZDF rats, concomitant with lowering of blood glucose.

4   The hyperinsulinaemic-euglycaemic clamp technique was applied to all rats after treatment to examine the effects of MCC-555 on insulin sensitivity. The glucose infusion rate to maintain normoglycaemia was lower in diabetic than in non-diabetic rats, demonstrating reduced glucose entry into insulin-sensitive tissues in diabetic rats. Increased glucose infusion rates were required to maintain euglycaemia in treated diabetic rats, demonstrating increased insulin sensitivity in these animals.

5   In conclusion, chronic MCC-555 treatment of young ZDF rats displaying IGT attenuates the development of overt diabetes through improved insulin sensitivity and maintenance of β-cell function. MCC-555 may thus be beneficial in humans with IGT, to prevent or delay the progression of diabetes.

Keywords: ZDF rats; MCC-555; thiazolidinediones; diabetes; insulin; impaired glucose tolerance

Introduction

The thiazolidinediones, such as troglitazone (Nolan et al., 1994; Whitcomb & Saltiel, 1995) and MCC-555 (($\pm$)-5-[{6-(2-fluorobenzyl)oxy-2-naphthyl}methyl]-2,4-thiazolidinedione; Ishii et al., 1996; Upton et al., 1997), represent a novel class of antidiabetic compounds that improve insulin sensitivity in subjects with insulin-resistant states, including non-insulin dependent diabetes mellitus (NIDDM or type 2 diabetes) and impaired glucose tolerance (IGT), but have little or no insulin secretagogue activity (Saltiel & Olefsky, 1996). In the early stages of the development of NIDDM, insulin sensitive tissues, such as adipose tissue and skeletal muscle, become insulin resistant, leading to the development of IGT, which may occur over several years (DeFronzo, 1988). Increased insulin secretion initially compensates for insulin resistance and is generally able to prevent the development of severe hyperglycaemia (DeFronzo, 1988). However, for reasons that are not fully understood, pancreatic β-cells ultimately become 'exhausted', and insulin secretion falls to normal or lower levels, which, in the presence of insulin resistance, allows glucose levels to rise into the range of overt diabetes (Harris, 1996; Polonsky et al., 1996). NIDDM is characterized by hyperglycaemia and osmotic symptoms (polydipsia and polyuria) and carries an increased risk of cardiovascular damage, especially when combined with the commonly associated high blood pressure and dyslipidaemia (Williams, 1994). Recent clinical studies have suggested that early intervention with troglitazone therapy in subjects with IGT can delay the onset or attenuate the development of overt diabetes (Nolan et al., 1994). We have therefore examined the effect of a novel thiazolidinedione, MCC-555, in an animal model of insulin resistance and NIDDM, namely the Zucker Diabetic Fatty (ZDF) rat. This mutant exhibits IGT at an early age, with moderate hyperglycaemia, insulin resistance and hyperinsulinaemia, and subsequently progresses to overt diabetes through a failure of β-cell function (Clark & Palmer, 1982; Peterson, 1994). The diabetes-prone ZDF strain is derived from the obese (fa^{-1}/fa) Zucker rat; both have the primary genetic defect of the Gln 279 Pro (fa) mutation affecting the extracellular domain of the leptin receptor, which results in impaired leptin signalling, and early-onset obesity attributable to both hyperphagia and reduced thermogenesis (Terrettaz & Jeanrenaud, 1983).

Experiments were performed to determine whether MCC-555 treatment of young IGT ZDF rats could prevent the development of diabetes in this genetic model of NIDDM.

Methods

Six-week-old male ZDF pre-diabetic (240 g) and non-diabetes prone ZDF controls (200 g) (Genetic Models Inc., Indianapolis, IN, U.S.A.) were divided into three groups ($n=9$ each). Rats were housed individually and kept at $22 \pm 2°C$ on a 12-h light/dark cycle (lights on at 07 00 h) and with free access to standard rodent chow (CRM, Biosure, Cambridge, U.K.) and tap water. One group of pre-diabetic ZDF rats received daily oral doses of MCC-555 [10 mg kg^{-1} (Mitsubishi Chemical

[3]Author for correspondence at: Diabetes and Endocrinology Research Group, Department of Medicine, UCD, Duncan Building, Daulby Street, University of Liverpool, Liverpool L69 3GA.

Corporation, Yokahama, Japan)], suspended in 0.5% sodium carboxymethycellulose vehicle (Sigma Chemical Company, Poole, Dorset, U.K.), for 28 days, whilst the other pre-diabetic rats were given vehicle alone (5 ml kg^{-1} d^{-1}). A group of non-diabetic rats also received daily oral doses of vehicle.

Body weight and food and water intake were measured daily, and every 4 days, tail-vein blood glucose concentrations were measured under the fed state, using an Exactech electrochemical meter (Medisense, Abingdon, Oxon, U.K.). At the end of each week, animals were lightly anaesthetized with i.p. injections of Diazemuls:Hypnorm:water mixture (1:1:2; Diazemuls: Dumex Ltd., Tring, Herts., U.K.; Hypnorm: Janssen Pharmaceutical Ltd., Oxford, U.K.) and 200 μl of tail-vein blood collected and placed in cooled microfuge tubes. The blood was immediately centrifuged (14,000 r.p.m. for 3 min) and 100 μl of plasma frozen for the later measurement of plasma insulin concentrations.

After 28 days, rats were anaesthetized with pentobarbitone (30 mg kg^{-1}; Sagatal: Harlow, Essex, U.K.) and their insulin sensitivity measured using the hyperinsulinaemic-euglycaemic clamp technique, as previously described (Terretaz & Jeanrenaud, 1983; Upton et al., 1997). Human soluble insulin (Humulin-S, Eli Lilly and Company, Basingstoke, Hants, U.K.) in isotonic saline containing 1% (w/v) bovine serum albumin (Sigma Chemical Co.) was infused at a constant flow rate of 14 mU min^{-1} into the right jugular vein to produce hyperinsulinaemia. A 5% glucose solution in isotonic saline was co-infused with the insulin at variable rates to maintain euglycaemia. All rats were also infused with 6-[^{3}H]glucose (0.2 mCi min^{-1}; Amersham International, Little Chalfont, Bucks., U.K.) prior to the clamping procedure, as described previously (Upton et al., 1997), in order to measure whole-body glucose uptake and hepatic glucose production. At the end of the clamp, rats were sacrificed by cardiac exsanguination and the perirenal and epidydimal (gonadal) fat pads dissected free and weighed. Final plasma glucose concentrations were measured using a standard kit (Boehringer Mannheim, Milton Keynes, Bucks., U.K.), and plasma insulin concentrations were measured using a radioimmunoassay kit (Pharmacia/Upjohn Diagnostics U.K., Lewes, Sussex, U.K.).

Statistical analyses

Differences in body weight, daily food and water intake, plasma insulin, blood glucose and glucose infusion rates during clamping between MCC-555-treated diabetic vehicle-treated diabetic and vehicle-treated non-diabetic rats were analysed using 2-way analysis of variance followed by Bonferroni modified t-tests for multiple comparisons.

Results

At the start of the experiment, pre-diabetic ZDF rats had significantly greater body weight, daily food intake and plasma insulin concentrations than non-diabetic controls (all $P<0.0001$; Figures 1 and 2). Pre-diabetic ZDF rats also displayed moderate hyperglycaemia, as compared to non-diabetic rats ($P<0.05$; Figure 2). At the end of the second week of the study, plasma insulin concentrations in the vehicle-treated pre-diabetic rats began to fall, as compared to their levels at the start of the study, becoming significantly lower by 32% and 52% at the end of weeks 3 and 4, respectively ($P<0.01$; Figure 2). These animals also became severely

hyperglycaemic and polydipsic by day 10, as compared to vehicle-treated non-diabetic rats ($P<0.0001$ and $P<0.01$, respectively; Figure 2). As a result of the development of overt diabetic symptoms, the body weight gain in vehicle-treated diabetic rats was blunted by day 14, although the rats remained significantly heavier than non-diabetic control rats throughout the study ($P<0.0001$; Figure 1).

In contrast to vehicle-treated pre-diabetic rats, MCC-555-treated pre-diabetic rats maintained initial plasma insulin concentrations throughout the study ($P<0.01$). Concomitantly, the rise in hyperglycaemia and polydipsia in MCC-555-treated rats was attenuated, as compared with vehicle-treated diabetic rats (both $P<0.01$; Figure 2). MCC-555-treated diabetic rats continued to gain weight throughout the study, as did vehicle-treated non-diabetic rats, but MCC-555 did not alter daily food intake in diabetic rats, as compared with vehicle-treated diabetic rats ($P>0.05$; Figure 1). Gonadal fat pad mass was significantly greater in MCC-555-treated diabetic rats, as compared with vehicle-treated diabetic rats at the end of the study ($P<0.01$), whilst both gonadal and perirenal fat pad masses were significantly greater in both diabetic rat groups than in the non-diabetic rats ($P<0.01$; Figure 3).

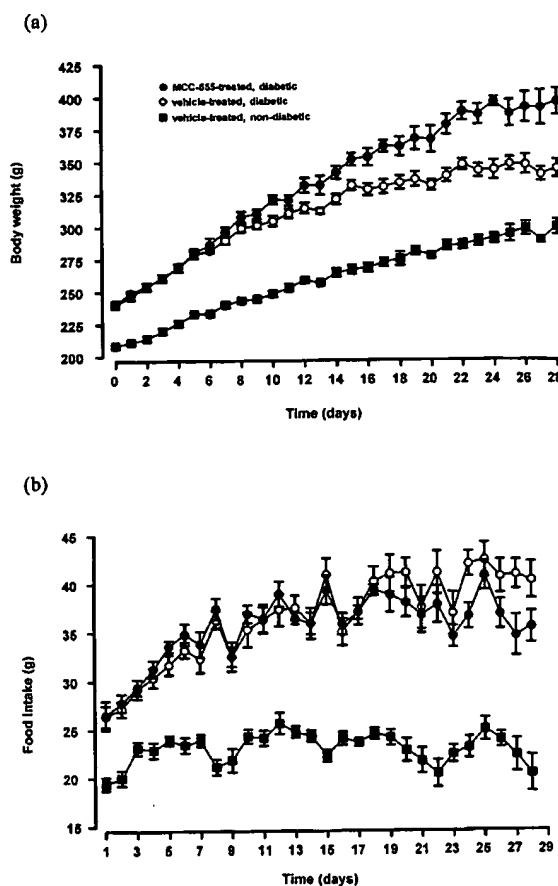Steady-state final glucose infusion rates during the hyperinsulinaemic-euglycaemic clamp were significantly greater in

Figure 1 (a) Body weight (g) and (b) daily food consumption (g) in vehicle-treated IGT ZDF rats, MCC-555-treated IGT rats and vehicle-treated non-IGT rats over the 28-day experimental period. Data are shown as means±s.e.mean for groups of nine rats.

the MCC-555-treated than in the vehicle-treated diabetic rats (diabetic = 0.06 ± 0.03 mg min^{-1}; MCC-555-treated = 0.72 ± 0.22 mg min^{-1}; $P < 0.01$), demonstrating significant improvements in glucose sensitivity under hyperinsulinaemic conditions ($+ 10.6\%$; $P < 0.05$).

Basal hepatic glucose production (HGP) and whole-body glucose uptake (WBU) rates were significantly greater in diabetic ZDF rats, as compared to lean rats ($P < 0.0001$). During the clamp, there was a significant suppression of HGP in both vehicle-treated lean and MCC-555-treated diabetic rats (both $P < 0.01$). Under clamp conditions, HGP and WBU in vehicle-treated diabetic rats did not alter significantly from basal levels ($P > 0.05$; Figure 4).
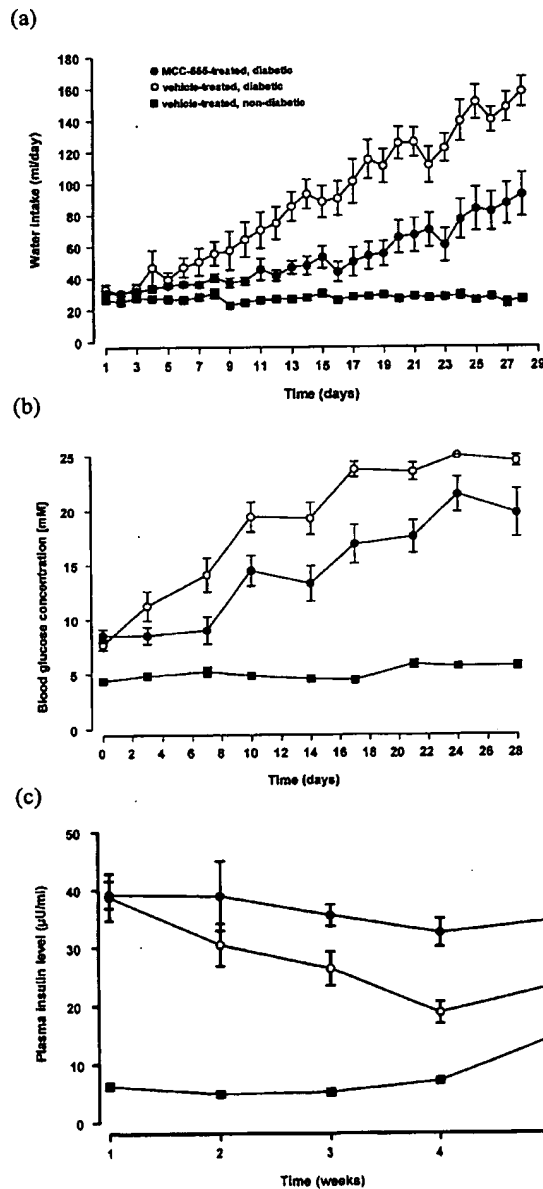
(a)

(b)

(c)

Figure 2 (a) Daily water intake (ml), (b) blood glucose (mmol l^{-1}) and (c) plasma insulin (μU ml^{-1}) concentrations in vehicle-treated IGT ZDF rats, MCC-555-treated IGT rats and vehicle-treated non-IGT rats over the 28-day experimental period. Data are shown as means $\pm$ s.e.mean for groups of nine rats.
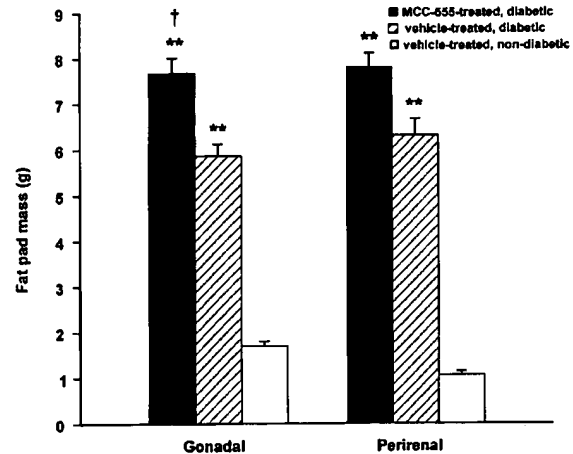
Figure 3 Gonadal and perirenal fat pad mass in MCC-555-treated diabetic rats, vehicle-treated diabetic rats and non-diabetic rats. Data shown as means $\pm$ s.e.mean for $n = 9$. **$P < 0.01$ as compared to non-diabetic rats; †$P < 0.05$ as compared to vehicle-treated diabetic rats.
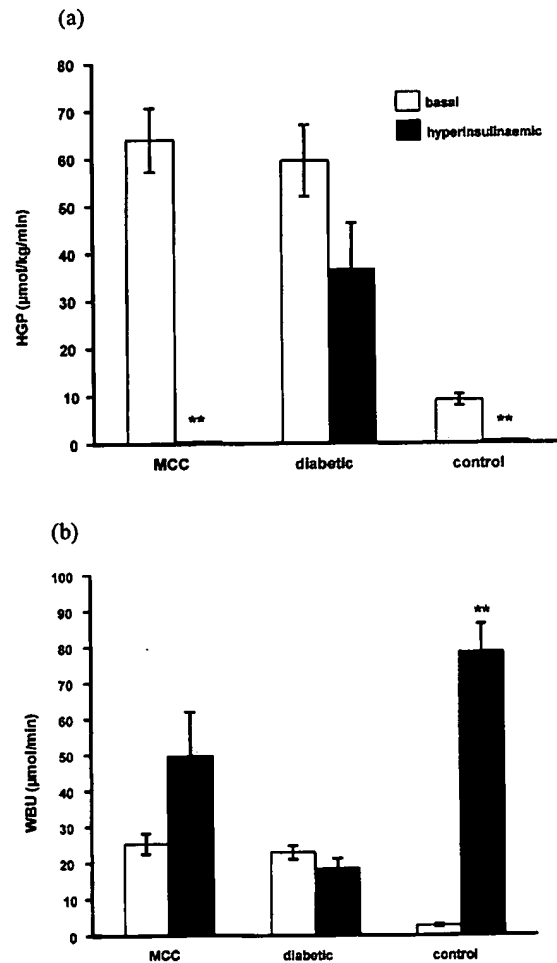
(a)

(b)

Figure 4 (a) Hepatic glucose production (HGP) and (b) whole body glucose uptake (WBU) in vehicle-treated IGT ZDF rats, MCC-555-treated IGT rats and vehicle-treated non-IGT rats under basal and hyperinsulinaemic conditions after 28 days of treatment. Data are shown as means $\pm$ s.e.mean for groups of nine rats. **$P < 0.01$ as compared to basal HGP or WBU.

Discussion

These data demonstrate that, as described previously (Clark & Palmer, 1982; Peterson, 1994), pre-diabetic ZDF rats displaying hyperinsulinaemia and moderate hyperglycaemia progress to β-cell failure and overt diabetes between 6 and 10 weeks of age. Overt diabetes is characterized by the development of severe hyperglycaemia (>20 mmol l^{-1}), polydipsia and attenuated body weight gain. We have also demonstrated that these diabetic ZDF rats have a reduced insulin sensitivity, as compared to their non-diabetic counterparts, as described previously in older diabetic ZDF rats (Upton *et al.*, 1997).

MCC-555 treatment of young pre-diabetic rats, which display IGT, significantly improved insulin sensitivity, as shown by the hyperinsulinaemic-euglycaemic clamp technique. Improved metabolic status was evident in the return of insulin-sensitive inhibition of hepatic gluconeogenesis observed under hyperinsulinaemic-euglycaemic clamp conditions in the drug-treated group. This has also been shown previously in older diabetic ZDF rats (Upton *et al.*, 1997). As a result of the sustained hyperinsulinaemia in the pre-diabetic MCC-555-treated rats, coupled with a partial restoration in insulin sensitivity, the development of overt diabetes was significantly attenuated in these rats, as demonstrated by the lower blood glucose levels, reduced polydipsia and continued increase in body weight gain and maintenance of body fat levels. The significant increase in gonadal fat pad mass in MCC-555-treated ZDF rats, as compared to vehicle-treated diabetic ZDF

rats, is probably a consequence of three processes. Firstly, the partial restoration in insulin sensitivity results in increased glucose entry into adipose tissue with subsequent conversion to triglycerides. Secondly, there is attenuation of the lipolysis resulting from catabolic metabolic processes associated with overt diabetes. Thirdly, thiazolidinedione compounds are selective agonists for peroxisome proliferator-activated receptor gamma (PPARγ; Forman *et al.*, 1995; Lehmann *et al.*, 1995), through which they induce adipogenesis by regulating expression of adipocyte-specific genes (Harris & Kletzien, 1994). MCC-555-mediated attenuation of the overt diabetic symptoms, including the development of severe hyperglycaemia in young ZDF rats, has also been reported following treatment with two other thiazolidinediones, troglitazone (Sreenan *et al.*, 1996) and BRL 49653 (rosiglitazone; Smith *et al.*, 1997).

In conclusion, we have demonstrated that chronic treatment of young ZDF rats displaying IGT with the thiazolidinedione MCC-555 can attenuate the symptoms of overt diabetes through a combination of partial restoration of insulin sensitivity and maintenance of β-cell function. The probable mechanism for the maintenance of insulin levels is the alleviation of glucose toxicity (see Yki-Järvinen, 1997); i.e., lowering glucose will non-specifically improve β-cell function (which is impaired by high blood glucose). We suggest that MCC-555 may be of significant potential therapeutic value in humans with IGT in preventing or attenuating the development of type 2 diabetes, as has been demonstrated for troglitazone (Nolan *et al.*, 1994).

References

CLARK, J.B. & PALMER, C.J. (1982). The diabetic Zucker rat — a new model for non-insulin dependent diabetes. *Diabetes*, **30**, 126A.

DEFRONZO, R. (1988). The triumvirate: beta cell, muscle, liver. A collusion responsible for NIDDM. *Diabetes*, **37**, 667–685.

FORMAN, B.M., TONTONOZ, P., CHEN, J., BRUN, R.P., SPIEGEL-MAN, B.M. & EVANS, R.M. (1995). 15-Deoxy-Δ12, 14 prostaglandin J2 is a ligand for the adipocyte determination factor PPARγ. *Cell*, **83**, 803–812.

HARRIS, M.L. (1996). Impaired glucose tolerance — prevalence and conversion to NIDDM. *Diabetes Med.*, **13** (3 suppl 2), S9–S11.

HARRIS, P.K. & KLETZIEN, R.F. (1994). Localization of a pioglitazone response element in the adipocyte fatty acid-binding protein gene. *Mol. Pharmacol.*, **45**, 439–445.

ISHII, S., WASAKI, M., OHNE, T., UENO, H. & TANAKA, H. (1996). MCC-555: A highly potent thiazolidinedione lacking hematological and cardiac side-effects. *Diabetes*, **45** (suppl 2), P141A.

LEHMANN, J.M., MOORE, L.B., SMITH-OLIVER, T.A., WILKISON, W.O., WILLSON, T.M. & KLIEWER, S.A. (1995). An antidiabetic thiazolidinedione is a high-affinity ligand for peroxisome proliferator-activated receptorγ (PPARγ). *J. Biol. Chem.*, **270**, 12953–12956.

NOLAN, J.J., LUDVIK, B., BEERDSEN, P., JOYCE, M. & OLEFSKY, J. (1994). Improvement in glucose tolerance and insulin resistance in obese subjects treated with troglitazone. *N. Engl. J. Med.*, **331**, 1188–1193.

PETERSON, R.G. (1994). The Zucker diabetic fatty (ZDF) rat. In *Lessons from animal diabetes*. ed. E. Shafrir. pp. 225–230. London: Smith-Gordon.

POLONSKY, K.S., STURIS, J. & BELL, G.I. (1996). Non-insulin-dependent diabetes mellitus — a genetically programmed failure of the beta cell to compensate for insulin resistance. *New Engl. J. Med.*, **334**, 777–783.

SALTIEL, A.R. & OLEFSKY, J.M. (1996). Thiazolidinediones in the treatment of insulin resistance and type II diabetes. *Diabetes*, **45**, 1661–1669.

SMITH, S.A., LISTER, C.A., HUGHES, M.G. & BUCKINGHAM, R.E. (1997). The PPARγ agonist, BRL 49653, prevents progression to diabetes in Zucker diabetic fatty rats. *Diabetologia*, **4** (suppl 1), A46.

SREENAN, S., STURGIS, J., PUGH, W., BURANT, C.F. & POLONSKY, K.S. (1996). Prevention of hyperglycemia in the Zucker diabetic fatty rat by treatment with metformin or troglitazone. *Am. J. Physiol.*, **271**, E742–E747.

TERRETTAZ, J. & JEANRENAUD, B. (1983). In vivo hepatic and peripheral insulin resistance in genetically obese (*fa/fa*) rats. *Endocrinology*, **112**, 1346–1351.

UPTON, R., WIDDOWSON, P.S., KADOWAKI, S. & WILLIAMS, G. (1997). Improved insulin sensitivity in Zucker and ZDF rats following chronic treatment with the novel thiazolidinedione, MCC-555. *Brit. J. Pharmacol.*, **122**, 149P.

WHITCOMB, R.W. & SALTIEL, A.R. (1995). Oncologic, endocrine and metabolic: thiazolidinediones. *Exp. Opin. Invest. Drugs*, **4**, 1299–1309.

WILLIAMS, G. (1994). Management of NIDDM. *Lancet*, **343**, 95–100.

YKI-JÄRVINEN, H. & WILLIAMS, G. (1997). Insulin resistance in non-insulin-dependent diabetes mellitus. In *Textbook of diabetes*. eds. J. Pickup & G. Williams. pp. 20.1–20.13. Oxford: Blackwell Science Ltd.